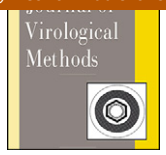
Journal of Virological Methods 186 (2012) 189–192

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Short communication

Optimization of one-step duplex real-time RT-PCR for detection of influenza and respiratory syncytial virus in nasopharyngeal aspirates

Fernanda de-Paris[a,b,*], Caroline Beck[c], Alice Beatriz Mombach Pinheiro Machado[a], Rodrigo Minuto Paiva[a], Denise da Silva Menezes[a], Luciana de Souza Nunes[b], Ricardo Kuchenbecker[c], Afonso Luís Barth[a,b]

[a] *Hospital de Clínicas de Porto Alegre (HCPA), Serviço de Patologia Clínica, Unidade de Pesquisa Biomédica – Laboratório de Biologia Molecular, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903, Brazil*

[b] *Universidade Federal do Rio Grande do Sul (UFRGS), Faculdade de Medicina, Programa de Pós-graduação em Ciências Médicas, Rua Ramiro Barcelos 2400, Porto Alegre, RS 90035-903, Brazil*

[c] *Instituto de Avaliação de Tecnologia em Saúde/IATS/CNPq, Universidade Federal do Rio Grande do Sul (UFRGS), Faculdade de Medicina, Programa de Pós-graduação em Epidemiologia, Rua Ramiro Barcelos 2400, Porto Alegre, RS 90035-903, Brazil*

Article history:
Received 2 May 2012
Received in revised form 28 June 2012
Accepted 4 July 2012
Available online 13 July 2012

Keywords:
Respiratory syncytial virus
Influenza virus
One-step real-time PCR
Molecular diagnostics
Molecular epidemiology

A B S T R A C T

Viruses are major contributors to acute respiratory infection-related morbidity and mortality worldwide. The influenza (IF) viruses and human respiratory syncytial virus (RSV) play a particularly important role in the etiology of acute respiratory infections. This study sought to standardize a one-step duplex real-time RT-PCR technique to optimize diagnosis of IFA/IFB and RSVA/RSVB infection. Viral RNA was extracted with the commercially available QIAamp Mini Kit according to manufacturer instructions. RT-PCR was performed with primers to the matrix protein gene of IFA, the hemagglutinin gene of IFB and the N gene of RSVA and RSVB. The limits of detection were 1 copy/μL for IFA, 10 copies/μL for IFB, 5 copies/μL for RSVA, and 250 copies/μL for RSVB. The specificity of RT-PCR was determined by comparison against a panel of several respiratory pathogens. RT-PCR and indirect immunofluorescence (IIF) were compared in a sample of 250 nasopharyngeal aspirates (NPAs) collected during the year 2010. RT-PCR was more sensitive than IIF and able to detect viral co-infections. In summary, RT-PCR optimized for IFA/IFB and RSVA/RSVB is sensitive and specific for these viral agents and is therefore useful for assessment of the etiology of respiratory infections, whether for clinical or epidemiological purposes.

1. Introduction

Viruses are major contributors to the morbidity and mortality of acute respiratory infections worldwide (Fairchok et al., 2010). Among these viruses, influenza (IF) and respiratory syncytial virus (RSV) are some of the most common etiological agents of respiratory infections. RSV is a well-known cause of lower respiratory tract infection in young children (Hall et al., 2009) and is now recognized as an increasingly important cause of respiratory infection in adults (Falsey et al., 2005; Caram et al., 2009). Influenza viruses remain significant causes of respiratory infections annually, despite the availability of vaccines and increasing efforts to achieve targeted vaccination rates (Kehl and Kumar, 2009). Furthermore, new IF strains have been described recently as etiological viruses in epidemics (Gatherer, 2009).

Accurate detection of respiratory viruses is important to guide antiviral therapy, to prevent nosocomial transmission during the seasonal period of more hospitalizations, and, in some cases, to decrease the hospital healthcare-associated costs (Kuypers et al., 2006). From a clinical standpoint, accurate identification of the causative agent of respiratory tract infections is important for proper clinical management. The identification of viruses is even more important for epidemiological purposes, such as the surveillance of respiratory infections during seasonal epidemics, assessment of high-risk groups, and allocation of hospital resources. On the other hand, diagnosis can be problematic, as a wide range of pathogens can cause acute respiratory infections presenting with similar clinical syndromes. Nucleic acid amplification testing is emerging as the preferred method of diagnostic testing for viral respiratory infections. Real-time technology and the ability to perform multiplex testing have facilitated the use of this molecular technique to diagnose a variety of respiratory viruses (Panning et al., 2009; Kehl and Kumar, 2009; Olofsson et al., 2011). This

* Corresponding author at: Hospital de Clínicas de Porto Alegre, Unidade de Microbiologia e Biologia Molecular, Rua Ramiro Barcelos 2350, Porto Alegre, RS 90035-903, Brazil. Tel.: +55 51 3359 8860; fax: +55 51 3359 8310.

E-mail addresses: fparis@hcpa.ufrgs.br, fparis@terra.com.br (F. de-Paris), beck.caroline@gmail.com (C. Beck), abmachado@hcpa.ufrgs.br (A.B.M.P. Machado), rpaiva@hcpa.ufrgs.br (R.M. Paiva), dmenezes@hcpa.ufrgs.br (D. da Silva Menezes), lucianadesnunes@hotmail.com (L. de Souza Nunes), rkuchen@gmail.com (R. Kuchenbecker), albarth@hcpa.ufrgs.br (A.L. Barth).

0166-0934 © 2012 Elsevier B.V. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.jviromet.2012.07.008

Table 1
"*In silico*" coverage of the sets of primers and probes against sequences obtained from GenBank using BioNumerics.

Viruses	Oligo	Primer/probe	Origin	Sequences	Possible identity	Identity	Gaps	Coverage
Flu A								
	InfA F	5′ GACCRATCCTGTCACCTCTGAC	CDC (2009)	131	2882	2813	69	97.6%
	InfA R	5′ AGGGCATTYTGGACAAAKCGTCTA	CDC (2009)	131	3144	3144	0	100%
	InfA Probe	5′ FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ	CDC (2009)	131	3144	3144	0	100%
Total coverage								99.24%
Flu B								
	BHA-188	5′ AR[a]ACCAGAGCGGAAACTATGCCC	Schweiger et al. (2000)	154	3388	3324	64	98.11%
	BHA-347	5′ CTGTCGTGCATTATAGGAAAGCAC	Schweiger et al. (2000)	154	3696	3671	25	99.32%
	BHA-273	5′ VIC-ACCY[a]TCGGCAARAGY[a]TTCAATACTCCA–TAMRA	Schweiger et al. (2000)	154	4158	4057	101	97.57%
Total coverage								98.31%
RSV A	A21	5′ GCTCTTAGCAAAGTCAAGTTGAATGA	Hu et al. (2003)	10	260	260	0	100%
	RSV compl	5′ AACATGCCACATAACTTATTGAT	This study	10	230	225	5	97.83%
	APB48	5′ FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC-TAMRA	Hu et al. (2003)	10	330	327	3	99.09%
Total coverage								98.97%
RSV B	B17	5′ GATGGCTCTTAGCAAAGTCAAGTTAA	Hu et al. (2003)	8	208	207	1	99.52%
	B120	5′ TGTCAATATTATCTCCTGTACTACGTTGAA	Hu et al. (2003)	8	240	238	2	99.17%
	BPB45	5′ VIC-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-TAMRA	Hu et al. (2003)	8	280	279	1	99.64%
Total coverage								99.44%

Degenerate nucleotides: R = A or G; Y = C or T, and K = G or T. Fluorophores: FAM (6-carboxyfluorescein) and VIC (6-carboxyrhodamine). Quenchers: BHQ (Black hole quencher) and TAMRA (6-carboxy-tetramethyl-rhodamine). Possible identity = no. of sequences obtained from GenBank × no. of nucleotides in primer or probe. Identity = no. of matches between nucleotides in primer or probe and nucleotides of sequences from GenBank. Gap = no. of mismatches between nucleotides in primer or probe and nucleotides of sequences from GenBank.
[a] Oligonucleotides degenerate in relation the original form reported by Schweiger et al. (2000).

study sought to validate a one-step duplex real-time polymerase chain reaction (RT-PCR) technique with the objective of optimizing diagnosis of IFA/IFB and RSVA/RSVB infection for clinical and epidemiological purposes.

2. Methods

2.1. Primer and probe design

The sequences of primers and probes, their original studies, and the results of *in silico* evaluation are shown in Table 1. RT-PCR was performed with primers specific to the matrix protein gene of IFA, the hemagglutinin of IFB, and the N gene of RSVA and RSVB (Hu et al., 2003; Schweiger et al., 2000; WHO, 2009).

2.2. RNA extraction and amplification conditions

RNA was extracted with the commercially available QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer instructions. Amplification was performed with the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA) in a 7500 Real Time PCR cycler (Applied Biosystems, Foster City, CA, USA). The amplification protocol was 50 °C for 30 min; 95 °C for 2 min; and 45 cycles of 94 °C, 15 s, and 55 °C, 35 s. Primers (Invitrogen, Carlsbad, CA, USA) at 800 nM and probes (Applied Biosystems, Foster City, CA, USA) at 200 nM were used for two different reactions, IFA/IFB amplification and RSVA/RSVB amplification. Positive controls included Fluvac® (Innovator EWT, Fort Dodge, IA, USA) for IFA and clinical samples for IFB, RSVA, and RSVB, which were confirmed by sequencing. Negative controls (sterilized water) were used in each reaction. In clinical samples, detection of a human gene (RNase P) was used as the internal control for the reaction. The reaction volume was 15 μL – 5 μL of RNA and 10 μL of reagent mix.

Primer and probe sets were tested first as a single reaction, then as two duplex reactions (RSVA/RSVB and IFA/IFB detection). Samples were considered positive if the Ct (Cycle threshold) value was $\leq$45 and the shape of the amplification curve was appropriate. Samples were considered negative if the internal control (RNAse P) was positive and there were no characteristic amplification curves for viral targets.

2.3. Limit of detection and assay specificity

Analytical sensitivity (limit of detection, LOD) was established by quantitative evaluation of the amplification products of positive controls. These amplifications were purified by ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and quantified in a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The number of copies of each control was obtained and serial dilutions (from 1 to 10^5 copies/μL) were used to determine the LOD. LOD assays were performed in duplicate for separate and duplex reactions.

Specificity assays were performed using a set of amplification products of clinical isolates with positive results for different respiratory pathogens (*Bordetella pertussis*, *Bordetella parapertussis*, *Enterovirus*, *Adenovirus*, *Cytomegalovirus*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, *Herpes simplex*, *Streptococcus pneumoniae*, *Pneumocystis jiroveci*, *Chlamydia pneumoniae*, RSV, Parainfluenza-1, Parainfluenza-2, Parainfluenza-3, and Epstein-Barr virus).

2.4. Patient samples

Nasopharyngeal aspirate (NPA) specimens were collected in phosphate-buffered saline (PBS) and frozen at −80 °C, followed by

Table 2
Results from 250 NPA samples.

Samples (n = 250)	IIF RSV	IIF IFB	RT-PCR RSVA	RT-PCR RSVB	RT-PCR IFB
112 (44.8%)	–	–	–	–	–
64 (25.6%)	+	–	+	–	–
35 (14%)	–	–	+	–	–
11 (4.4%)	+	–	–	+	–
11 (4.4%)	–	–	–	+	–
5 (2%)	+	–	–	–	–
3 (1.2%)	+	–	+	+	–
3 (1.2%)	–	+	–	–	+
2 (0.8%)	–	–	+	–	+
2 (0.8%)	+	–	+	–	+
1 (0.4%)	+	–	–	–	+
1 (0.4%)	–	–	–	–	+

IIF, immunofluorescence; RT-PCR, one step duplex RT-PCR; –, negative; +, positive.

RNA extraction and RT-PCR amplification. This study included 250 NPAs collected between May and September 2010 and previously tested for respiratory viruses by a commercially available indirect immunofluorescence (IIF) assay (Medivax®, Dublin, Ireland). A comparison between RT-PCR and IIF could thus be established. All NPAs had been obtained for clinical purposes as part of standard care. The study protocol was approved by the Institutional Review Board of Hospital de Clínicas de Porto Alegre.

3. Results

In silico coverage of the selected oligonucleotides was >98% (Table 1). This coverage rate was considerate adequate for use of this set of probes and primers.

The positive controls showed adequate results (Ct <20) in both protocol conditions (single and duplex reaction). The LOD was 1 copy/μL for IFA, 10 copies/μL for IFB, 5 copies/μL for RSVA, and 250 copies/μL for RSVB. These LODs were similar in separate and duplex reactions. LOD data showed a Poisson distribution and the corresponding obtained Cts showed linearity (data not shown).

All tests for different respiratory pathogens had negative results, which show an absence of cross-reactivity between RT-PCR and this panel.

Of the tested samples, 51.2% (128/250) were positive for RSV by RT-PCR, while only 34.3% (86/250) were positive by IIF. Furthermore, 3.6% (9/250) were positive for IFB by RT-PCR, while only 1.2% (3/250) were positive by IIF. No samples were positive for IFA with either technique. Among the clinical specimens positives, 89.7% showed Ct values between cycles 12 and 39.

In this study, IIF failed to detect RSV in 46 samples (35 for RSVA and 11 for RSVB) as compared with RT-PCR. RT-PCR failed to detect RSV in only five samples when compared with IIF. All three samples that tested positive for IFB on IIF were detected by RT-PCR. Furthermore, IIF did not detect IFB in six samples when compared with RT-PCR (Table 2). It should be noted that RT-PCR was able to detected co-infection (three RSVA/RSVB co-infections and four RSVA/IFB co-infections), whereas the IIF technique was not (Table 2).

4. Discussion

The aim of this study was the optimizing of a one-step duplex RT-PCR technique for IFA/IFB and RSVA/RSVB detection. Primers and probes were adapted from different protocols (Table 1) to establish a single assay with the same extraction and amplification conditions. This optimized assay proved specific for the viruses of interest, as no cross-reaction against a panel of other respiratory pathogens was detected. Furthermore, this assay exhibited high analytical sensitivity, being able to detect as low as 1 copy/μL of IFA, 10 copies/μL of IFB, 5 copies/μL of RSVA, and 250 copies/μL of RSVB.

The results of this study demonstrated that protocol performed well on clinical samples, resulting in an increasing the detection of IFB and RSVA/RSVB in NPA specimens. The IIF assay detected 89 samples (35.6%) and RT-PCR detected 133 (53.2%), thus proving the higher sensitivity of RT-PCR assay as compared with IIF. This increased sensitivity could be explained by the ability of PCR methods to detect lower viral loads in clinical samples, as previously reported by Kuypers et al. (2006), who showed that the mean number of viral copies per mL in specimens positive on both PCR and IIF was significantly higher (10^7) than that of specimens positive only on PCR (10^4). This suggests that PCR is suitable for detection of respiratory viruses in samples from adults which often exhibit lower viral loads than samples obtained from children (Kuypers et al., 2006). Furthermore, it is known that probability of detection of respiratory viruses increases with the duration of symptoms. Patients presenting within 0–6 days of symptom from the onset exhibit less viral shedding than those tested 7–14 days after symptoms arise (Brittain-Long et al., 2010; Olofsson et al., 2011). Therefore, this optimized RT-PCR technique could improve the possibility of viral detection in samples collected longer after the onset of symptoms.

In addition, it bears stressing that all samples testing positive for IFB on IIF were detected by RT-PCR, and only five samples positive for RSV on IIF were not detected by RT-PCR. Remarkably, none of the specimens used in this study were positive for IFA by either method. This was most likely due to the extensive immunization campaign against influenza A/H1N1 and other seasonal influenza strains that was carried out worldwide, including in Brazil, in 2010.

Another important aspect of optimized RT-PCR is that this technique can be performed with frozen samples, unlike IIF, which requires fresh specimens. In a previous study, respiratory viruses were detected by PCR in 25% of specimens judged inadequate for IIF analysis (Kuypers et al., 2006).

Concerning viral co-infection, seven cases were found in this study (three co-infections with RSVA and RSVB and four co-infections with RSVA and IFB) by RT-PCR; none was detected with the IIF technique. Several studies have acknowledged the possibility of multiple infections in viral respiratory diseases detected by molecular methods (Kuypers et al., 2006; Paranhos-Baccalà et al., 2008). Furthermore, multiple respiratory viruses have been detected in approximately 10% of respiratory specimens, with higher rates recorded in samples from young children and immunosuppressed patients (Olofsson et al., 2011). Some authors suggest an association between dual infections and increased disease severity (Richard et al., 2008; Paranhos-Baccalà et al., 2008; Olofsson et al., 2011). Indeed, the clinical and epidemiological significance of co-infections is uncertain, but infection with multiple viruses can only be detected with the use of molecular techniques. Notably,

the optimized RT-PCR protocol described herein was able to detect RSVA and RSVB separately, which may be very useful for studies with epidemiological purposes.

Furthermore, this study has considered that the real time PCR technique is suitable for limited multiplexing. This technique is also excellent for in-house testing, providing an affordable analysis capability for the outpatient clinic (Olofsson et al., 2011). Therefore, this RT-PCR technique could be feasible and more cost-effective for routine clinical than a multiplex 15-virus panel or other extended molecular diagnostics.

In summary, the one-step duplex RT-PCR technique optimized for IFA/IFB and RSVA/RSVB described herein is suitable for the clinical laboratory setting, requiring only one RNA extraction and using the same amplification conditions as used for multiple virus detection, as well as internal controls. It is less labor-intensive than IIF and exhibited reliable sensitivity and specificity, making it useful for establishment of the etiology of respiratory infections for clinical and epidemiological purposes alike.

Acknowledgments

This study was supported by Fundo de Incentivo à Pesquisa e Ensino, Hospital de Clinicas de Porto Alegre (FIPE/HCPA), the Rio Grande do Sul Research Foundation (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul, FAPERGS), and the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq).

References

Brittain-Long, R., Westin, J., Olofsson, S., Lindh, M., Andersson, L.M., 2010. Prospective evaluation of a novel multiplex real-time PCR assay for detection of fifteen respiratory pathogens-duration of symptoms significantly affects detection rate. Journal of Clinical Virology 47, 263–267.

Caram, L.B., Chen, J., Taggart, E.W., Hillyard, D.R., She, R., Polage, C.R., Twersky, J., Schmader, K., Petti, C.A., Woods, C.W., 2009. Respiratory syncytial virus outbreak in a long-term care facility detected using reverse transcriptase polymerase chain reaction: an argument for real-time detection methods. Journal of the American Geriatrics Society 57, 482–485.

Fairchok, M.P., Martin, E.T., Chambers, S., Kuypers, J., Behrens, M., Braun, L.E., Englund, J.A., 2010. Epidemiology of viral respiratory tract infections in a prospective cohort of infants and toddlers attending daycare. Journal of Clinical Virology 49, 16–20.

Falsey, A.R., Hennessey, P.A., Formica, M.A., Cox, C., Walsh, E.E., 2005. Respiratory syncytial virus infection in elderly and high-risk adults. New England Journal of Medicine 352, 1749–1759.

Gatherer, D., 2009. The 2009 H1N1 influenza outbreak in its historical context. Journal of Clinical Virology 45, 174–178.

Hall, C.B., Weinberg, G.A., Iwane, M.K., Blumkin, A.K., Edwards, K.M., Staat, M.A., Auinger, P., Griffin, M.R., Poehling, K.A., Erdman, D., Grijalva, C.G., Zhu, Y., Szilagyi, P., 2009. The burden of respiratory syncytial virus infection in young children. New England Journal of Medicine 360, 588–598.

Hu, A., Colella, M., Tam, J.S., Rappaport, R., Cheng, S., 2003. Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. Journal of Clinical Microbiology 41, 149–154.

Kehl, S.C., Kumar, S., 2009. Utilization of nucleic acid amplification assays for the detection of respiratory viruses. Clinics in Laboratory Medicine 29, 661–671.

Kuypers, J., Wright, N., Ferrenberg, J., Huang, M.L., Cent, A., Corey, L., Morrow, R., 2006. Comparison of real-time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children. Journal of Clinical Microbiology 44, 2382–2388.

Olofsson, S., Brittain-Long, R., Andersson, L.M., Westin, J., Lindh, M., 2011. PCR for detection of respiratory viruses: seasonal variations of virus infections. Expert Review of Anti Infective Therapy 9, 615–626.

Panning, M., Eickmann, M., Landt, O., Monazahian, M., Olschläger, S., Baumgarte, S., Reischl, U., Wenzel, J.J., Niller, H.H., Günther, S., Hollmann, B., Huzly, D., Drexler, J.F., Helmer, A., Becker, S., Matz, B., Eis-Hübinger, A., Drosten, C., 2009. Detection of influenza A (H1N1)v virus by real-time RT-PCR. Euro Surveillance 14, 1–6.

Paranhos-Baccalà, G., Komurian-Pradel, F., Richard, N., Vernet, G., Lina, B., Floret, D., 2008. Mixed respiratory virus infections. Journal of Clinical Virology 43, 407–410.

Richard, N., Komurian-Pradel, F., Javouhey, E., Perret, M., Rajoharison, A., Bagnaud, A., Billaud, G., Vernet, G., Lina, B., Floret, D., Paranhos-Baccalà, G., 2008. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. Pediatric Infectious Disease Journal 27, 213–217.

Schweiger, B., Zadow, I., Heckler, R., Timm, H., Pauli, G., 2000. Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. Journal of Clinical Microbiology 38, 1552–1558.

World Health Organization, 2009. CDC Protocol of Real Time RT-PCR for Swine Influenza A (H1N1). WHO, Atlanta.